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The relative effects of age on implant integration in a rat model: a longitudinal *in vivo* microCT study.

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Contents

1 Abstract

The effect of age on implant fixation in bone is not always considered during the design of preclinical models. The decision on animal's age is often related to practical or historical reasons, which ultimately may affect the reproducibility of results. This study aimed to quantify the effect of age by monitoring the fixation of contrast-enhanced PEEK screws in rats, hypothesizing that the kinetics of fixation is impaired in older animals but that age effects are less severe than osteoporotic effects.

The time course of implant fixation was investigated in healthy rats at 24, 40 and 60 weeks of age; and in ovariectomized rats. Implant fixation was monitored using in-vivo microCT for one month. The rats were euthanised 28 days post screw insertion. The data was analysed both in absolute value and after normalization to baseline bone mass. In the absolute data age had a detrimental effect on bone implant contact, bone fraction, implant stiffness and bone remodeling, but less than ovariectomy, but interestingly this effect disappeared once, data was normalized to baseline bone mass, suggesting that the physiologic reaction to implant placement was not affected by age, and that baseline bone mass was the main factor influencing implant fixation strength.

In conclusion, implant fixation kinetics is less affected by age than by baseline bone mass. Animals of different ages can therefore be compared but data must be construed relatively to baseline bone mass and not in absolute terms.

Keywords

Age, implant fixation, osseointegration, longitudinal in vivo microCT, rat, osteoporosis

2 Zusammenfassung

Der Alterseinfluss auf Implantatsfixation in Knochen wird bei der Planung von präklinischen Studien oft vernachlässigt. Der Entscheid bezüglich Alter der Tiere ist oft von Praktikabilität oder Historie abhängig, wobei es aber in direktem Zusammenhang zur Reproduzierbarkeit von Studien stehen dürfte. Ziel dieser Studie war, den Alterseffekt zu quantifizieren, indem die Fixation von kontrastmittelhaltigen PEEK-Schrauben in Ratten untersucht wurde. Wir hypothetisierten, dass die Fixations-Kinetik in älteren Tieren beeinträchtigt ist, wobei der osteoporotische Einfluss noch stärker ist.

Die Implantatsfixation wurde in gesunden Ratten im Alter von 24, 40 und 60 Wochen und in ovariektomierten Ratten untersucht und mittels in-vivo microCT überwacht. Nach 28 Tagen wurden sie euthanasiert.

Die Daten wurden absolut und nach Normalisierung zu Tag 0 analysiert. In den absoluten Werten hatte Alter einen nachteiligen Effekt auf Knochen-Implantat Kontakt, Knochenfraktion, -remodelling und Implantatstabilität, der bei Ovariektomie noch stärker ausfiel. Bei Normalisierung verschwand dieser Effekt, was die Vermutung nahelegt, dass die physiologische Reaktion auf das Implantat nicht durch Alter sondern durch Knochenmasse an Tag 0 beeinflusst wird.

Schlussfolgernd wird die Kinetik der Implantatsfixation weniger durch Alter sondern durch die Knochenmasse an Tag 0 beeinflusst. Tiere verschiedenen Alters können also nicht absolut sondern nur nach Normalisierung verglichen werden.

Schlüsselwörter

Alter, Implantatsfixation, Osseointegration, Longitudinales in-vivo microCT, Ratte, Osteoporose

3 Submitted Manuscript

The relative effects of age on implant integration in a rat model: a longitudinal *in vivo* microCT study.

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3.1 Abstract

The effect of age on implant fixation in bone is not always considered during the design of preclinical models. The decision on animal's age is often related to practical or historical reasons, which ultimately may affect the reproducibility of results. This study aimed to quantify the effect of age by monitoring the fixation of contrast-enhanced PEEK screws in rats, hypothesizing that the kinetics of fixation is impaired in older animals but that age effects are less severe than osteoporotic effects.

The time course of implant fixation was investigated in healthy rats at 24, 40 and 60 weeks of age; and in ovariectomized rats. Implant fixation was monitored using in-vivo microCT during one month. The rats were euthanised 28 days post screw insertion. The data was analysed both in absolute value and after normalization to baseline bone mass. In the absolute data age had a detrimental effect on bone implant contact, bone fraction, implant stiffness and bone remodeling, but less than ovariectomy, but interestingly this effect disappeared once, data was normalized to baseline bone mass, suggesting that the physiologic reaction to implant placement was not affected by age, and that baseline bone mass was the main factor influencing implant fixation strength.

In conclusion, implant fixation kinetics is less affected by age than by baseline bone mass. Animals of different ages can therefore be compared but data must be construed relatively to baseline bone mass and not in absolute terms.

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3.2 Introduction

Preclinical investigations are essential to evaluate the efficacy and safety of novel solutions to improve fixation of both, orthopedic and dental implants [1,2]. Alas Freedman et al. have reported that up to 50% of preclinical research performed in the US is irreproducible [3]. Numerous factors were identified as main causes leading to this reproducibility crisis: wrong choice of biological reagents or laboratory protocols, flawed study design or data analysis and incomplete reporting. In preclinical studies, Jackson et al. found that the choice and the reporting of animals age is still often lacking [4] despite its established influence on bone metabolism and healing [5].

In clinical practice, age is largely recognized as a complication factor for implant placement [6–8], and age and menopause count as risk factors for bone diseases in humans [9]. While the impact of osteoporosis on osseointegration and implant fixation in human bone and rodent research models has been well documented[10,11], the effect of age remains controversial in animals. Nilsson and Edwards initially reported a lack of correlation between bone healing time and age clinically [12], but it was later shown that age increases bridging times [13] and lower bridging scores, probably because of impaired bone response to loading and higher osteoclast numbers [14]. The root cause of this effect is still unclear, but may be related to cellular senescence and Wnt/ß-catenin signaling in hematopoietic cells [15].

In preclinical models, age is regularly overlooked and not reported with enough details. For practical reasons such as cost, time and supply or for comparability with previous reports, sexually mature rodents (8 to 12 weeks for mice, 5 to 10 weeks for rats)[16,17] are used most of the time when age is not the primary focus[4]. But skeletal maturity is reached later than sexual maturity[18]. This lack of study-adapted choice of animals' age might negatively impact research outcomes, since such young animals are still experiencing physiological changes [19].

Here, the aim of the study was to characterize the effects of age on the dynamics of implant fixation in a longitudinal rat model; and to compare it with the effect of osteoporosis. We hypothesized that the kinetics of screw fixation is impaired in older animals but that age effects are less severe than osteoporotic effects. Implant integration and osseointegration are in essence transient, time-dependent processes with primary and secondary phases[20,21]. Typically, investigations on the effect of age or drugs on implant fixation focus on one or two ending timepoints [22–24], with the risk of missing the phase transitions. To avoid this risk, we implanted PEEK mini-screws in rats of varying age and in ovariectomized rats and followed their evolution with longitudinal *in vivo* micro computed tomography (microCT).

3.3 Materials and methods

Study Design

Thirty skeletally mature female Wistar rats were randomly assigned into four groups (Table 1). Animals included in this analysis were used as control groups for other studies resulting in different group sizes. At age 24, 40±6, 60±5 weeks (groups 1—3 respectively), they received a contrast enhanced PEEK screw in the medio-proximal tibia and were longitudinally scanned with in vivo microCT over a time period of 4 weeks, at 0, 3, 6, 9, 14, 20 and 28 days post-operatively. These groups were compared to ovariectomized rats, which received the screw at 33 weeks (group 4).

As primary outcomes, the scan data were used to evaluate changes in bone fraction (BV/TV) and bone implant contact (BIC) in defined regions of interest around the screw, as previously described[25]. Secondary outcome measures included weight, apparent bone mineral density (BMD), tissue mineral density (TMD), bone formation (BF) and bone resorption (BR) and mechanical stiffness simulated by conducting micro finite element analysis to calculate the force necessary to pull out the screw from the implanted site in the tibia. All investigators were blinded for the data analysis.

Implants

Custom-made medical grade polyetheretherketone (PEEK) screws containing 20% Barium sulfate ($BaSO_4$), manufactured by RISystem (Davos, Switzerland) with an inner/outer diameter of 1.2/1.4mm and a threaded length of 2.7mm, were used in this project. This concentration of $BaSO_4$ gives a higher contrast than mature bone, but does not create metal artefacts in microCT images, like metallic screws would and are therefore ideal for longitudinal microCT studies. The implants were steam sterilized before surgery.

Animals

30 female, specific pathogens free (according to FELASA guidelines) Wistar rats were obtained from Charles River, Germany. The in-vivo study was approved by the ethical committee of the canton of Grisons, Switzerland, and performed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) accredited facility. The rats were housed in groups of N=3 in individually ventilated cages (type 2000, Allentown Inc, USA) under a positive pressure system. Wood chips served as bedding; cages were enriched with a cardboard house, paper tissues and a piece of wood. Environmental conditions were set for > 40% humidity, a temperature of 22±2°C and a 12h light/dark cycle. Sterilized cages were changed weekly; food (Alleinfuttermittel für Mäuse und Ratten, Provimi Kliba, Switzerland) and autoclaved tap water were provided ad libitum.

The 30 animals were divided in 4 groups (Table 1). Group 1 received the screw at age 24 weeks, group 2 at 40±6 and group 3 at 60±5 weeks. Group 4 was ovariectomized (OVX) at 13 weeks, followed by screw insertion at 33 weeks. The 9 months timepoint for treatment evaluation in ovariectomized rats was chosen based on to the suggestions by Jee *et al.* [26].

Anesthesia, Analgesia

The rats were placed in a Plexiglas chamber for anesthesia induction at 1000 ml/min oxygen and 3% isoflurane. Using a rodent facemask, eye ointment (Vitamin A Augensalbe®) and

heating pads, anesthesia was maintained at 400-500 ml/min oxygen and approx. 1.5 % isoflurane. After each anesthesia the animals were weighed.

Preemptively, the rats were injected with 0.01 mg/kg buprenorphine (Bupaq® ad us. vet.) and 4 mg/kg carprofen (Rimadyl®), both subcutaneously (sc). Within the first 24 hrs post op, the rats received two more injections of 0.01 mg/kg buprenorphine sc (7±1 and 14±1 hours post op). For the first 5 days postop, paracetamol was added to the drinking water (7 ml DAFALGAN Sirup®/ 100ml). Postoperatively, 10 ml/kg warm ringer solution was given intraperitoneally.

Surgery: Ovariectomy

Animals of the OVX group were ovariectomized at 13 weeks of age**.** After aseptic preparation and subsequent positioning in dorsal recumbency on a heating pat, the linea alba was incised, centered between the end of the sternum and the pubic symphysis. Lifting one uterine horn with a nerve hook, small vessel clamps secured the tip of the horn and the ovary. The ovary was then removed by electrocauterisation. After repetition of the procedure on the contralateral side, the incision was closed with a continuing adaptive suture pattern in two layers using resorbable suture material (Monocryl 4/0 and Vicryl rapide 5/0, Ethicon®).

Surgery: Screw Implantation

The rat was placed in dorsal recumbency on a heating pad and the tibia was aseptically prepared. A skin incision was made on the medio-proximal aspect of the right tibia and extended through the fascia down to the medial surface of the tibia. After identification of the proximal, tibial growth plate a unicortical hole was drilled by hand (1.2 mm drill bit; Figure 1 A) approximately 2 mm distal of the growth plate and in the centre of the medial aspect of the bone. A custom-made stop guide was used to avoid drilling into the far cortex. After tapping the thread, a 1.4/1.2 mm (outer/inner diameter) partially threaded screw was placed into the hole whereas the unthreaded part of the screw did not enter the cortex (Figure 1 B-D). After full insertion, the screw extension was clipped off by use of a custom-made screw clipper. The fascia and the

skin were closed in two layers using absorbable suture material (Monocryl 4/0 and Vicryl rapide 5/0, Ethicon®, respectively).

Animal welfare

Animals were checked daily. Objective welfare evaluation using a score sheet was performed daily for the first 7 days after surgery, then at time point of every microCT scan. Weight measurements were carried out at surgery time and before microCT scan.

Euthanasia

At day 28, all animals were anesthetized (induction: 3 % isoflurane in 1000 ml/min oxygen; maintenance: 1,5-2 % isoflurane, 400 ml/min oxygen prior to euthanasia by intracardiac injections of pentobarbital (180 mg/rat; Esconarkon®).

Imaging

In-vivo microCT

The animals were microCT scanned immediately postoperatively and afterwards at day 3, 6, 9, 14, 21 and 28 after surgery (VivaCT40, Scanco Medical AG, Bruettisellen). Anesthesia was induced and maintained as above, while the mask was integrated in a standard rat holder. The operated tibia was pulled to full extension of the stifle joint and the ankle was fixated in a position of approx. 90° flexion. The scanned region of 10 mm in length and ø25.6 mm field of view was centered on the implanted screw. The scanner was operated at 70 kV tension, 114 µA current, 220 ms integration time and 500 projections/180°. Scanning time was 21 min, and total anesthesia duration was 25-30 min per scan. Each scan resulted in a radiation dose of 200 mGy, which has no effect on metabolism[27]. Each scan consisted of 420 slices reconstructed across an image matrix size of 1024 x 1024 voxels, with an isotropic voxel size of 25 µm.

Image Processing

First, the post-op scans (d0) were aligned so that the screws' main axis was along the Z-axis of the image and the bone's main axis along the X-axis. The following scans were aligned to the postoperative scan via rigid registration (≥ 0.8 correlation coefficient, $1*10^{-5}$ convergence

criterion, max. 2000 iterations). The images were segmented with a bone threshold of 580 mgHA/cm^3 and a PEEK threshold of 1500 mgHA/cm^3.

Two regions of interest (ROI) were automatically generated by successive dilations: bone at implant surface (ROI 1) and peri-implant bone (ROI 2). ROI 1 margins were defined as the volume within 75 µm from the threaded screw surface. ROI 2 was defined as the volume within 75 to 700 µm from the threaded screw surface (Figure 2).

Bone-implant contact (BIC) was computed by voxel-counting of the segmented image within ROI 1 and bone fraction (BV/TV), apparent bone mineral density (BMD) and tissue mineral density (TMD) within ROI 2, using standard methods[28]. Bone formation (BF) and bone resorption (BR) were computed by "dynamic histomorhometry" by mapping differences in two subsequent registered scans [29,30] within ROI 2 (Figure 3). The image processing algorithms were developed with EasyIPL a high-level library of macros using the scanner software (Image Processing Language (IPL).

Finite Element Analysis: Screw Pull-out Simulation

Screw pull-out strength was estimated with micro-finite element analysis (microFE) at all time points. The voxel conversion approach with linear elastic analysis was used after preparation of the segmented data [31]. The settings were as following: isotropic Young's modulus with 69 GPa for screw and 6,8 GPa for bone and Poisson's ratio with 0.3 for both. Margins for uniaxial pullout simulation of the screw with 1 % displacement of the screw head were defined as a surface radius of 2 mm of the screw axis. All surfaces further away were fully constrained. The boundary conditions were defined as previously described [32] and the model was solved using Faim software 6.0 (Numerics88Solutions Ltd, Calgary, Canada) on a desktop workstation (MacPro, Apple Inc., Cupertino, CA, USA) for pullout stiffness (N/mm) and failure load (N) estimated with the "Pistoia criterion" [33].

Statistical analysis

Data is expressed as Mean ± SD. To account for differences at baseline, time series showing absolute values and time series showing values normalized to day 0 were produced for each outcome parameter. Outcomes marked with a * are normalized (for example "BIC" is absolute and "BIC*" is normalized). In the timeseries, data is shown as Mean ± SE for sake of readability of the plots. MANOVA was used to determine the significance of the effect of age and OVX on the measured outcomes. Then, the time series were fitted with a linear additive mixed model and the 95 % confidence intervals of the fitted curves computed to determine the time ranges where the groups were statistically different. Differences between means at the 5 % and 1 % confidence level ($p<0.05$, and $p<0.01$) were considered statistically significant and highly significant respectively. All analyses were performed in the R programming language (version 3.3.3) [34].

3.4 Results

Animal welfare

All animals listed in table 1 were included for analysis for all outcome measures.

In terms of visual aspect and behavior, all animals recovered fast from anesthesia and surgery, as well as from the regular anesthesia during microCT. In one animal of the 24w group, the wound opened one day after surgery and was re-sutured immediately, healed normally and was therefore included in the study.

A positive correlation between weight and age ($r = 0.78$, $p < 0.01$) was observed. At day 0, 33w OVX animals were heavier than 24w animals ($p < 0.01$). All animals lost between 1 and 20 % of weight in the three days following screw implantation. Age had a significant impact on post-operative weight loss (weight at day 3 compared to day 0), with younger animals losing relatively more weight than older ones ($p < 0.001$). Overall, the animals regained their initial weights at screw implantation during the 28 days observation period, without influence of age (Figure 4).

Qualitative evaluation of screw osseointegration

At baseline (day 0), bone density in the proximal tibia metaphysis was visually negatively correlated with age. The younger animal showed a progressive encapsulation of the screw by newly formed bone until day 14. This newly formed bone seems to undergo remodeling already between day 20 and 28. The encapsulation process seemed less marked in the 40 and 60 weeks groups. In comparison, the OVX animals showed no encapsulation (Figure 5).

Quantitative analysis with longitudinal microCT

All groups showed similar patterns in bone-implant contact (BIC) and bone fraction (BV/TV) evolution, with a continuous increase from day 0 to 9-14, followed by a decrease from day 14 to 28. In absolute terms, BIC was positively correlated with age ($p<0.01$). The 40w and 60w groups were significantly different from the 24w group from day 0 to 28. The 40w group was significantly different from 60w only from day 4 to 10. The 24w group reached its peak BV/TV value on day 9 and BV/TV on day 14, while the other groups reached peak values for both BIC and BV/TV on day 14. The effect of OVX alone on BIC was significant, too ($p < 0.001$) (Figure 6).

Interestingly, the normalized data showed different patterns. BIC* had a negative correlation with age. All groups showed equivalent increases from day 0 until day 9. Between day 9 and 14, the 24w group plateaued, while the 40w and 60w continued to increase resulting in a significantly higher BIC* from day 14 to 28 compared to 24w ($p < 0.05$). BV/TV* and Stiffness* showed the same patterns.

Stiffness was evaluated with micro finite elements. Both age and OVX had significant effects on stiffness. In 24w animals, it increased slightly from 7±1 to 8±1 N at day 9 and returned to baseline by day 20, suggesting the primary fixation of 7N was stable enough. The 40w and 60w animals had initially 43% lower stiffnesses compared to 24w ($p<0.01$) but by day 14 they had gained 50% stiffness at 6±0.6 N. OVX initial stiffness was only at 42% of the 24w and increase only until 50% of the 24w animals.

The general pattern of bone formation (BF) was similar for all groups (Figure 7): a rapid increase following surgery up until day 6 to 9, followed by a decrease back to baseline levels. BF peaked day 6 in rats of group 24w, earlier than in 40w, 60w and OVX which all peaked at day 9. The peak of OVX rats was also significantly lower than the others ($p < 0.05$). In the non-OVX groups, the absolute peak bone formation level correlated negatively with age ($p < 0.05$). Interestingly, BF* (normalized to baseline bone volume), showed no more differences between groups.

Bone resorption was highest at day 3 immediately post-surgery then decreased. A second moderate increase happened at day 20 in all groups. The OVX group had significantly lower BR on day 3, but higher on day 20 ($p<0.05$). The peak levels of bone formation and resorption significantly decreased with age and ovariectomy ($p < 0.01$**).

Net bone formation (BF-BR) peaked at day 9 in all groups, the peak level of the ovariectomized group was significantly lower. Besides of this, all groups behaved similar in the next CT scans and Net Bone Formation converged towards 0. Remodeling balance (BF/BR) showed no significant differences between groups (*Figure 7*).

Bone Mineral Density (BMD) data showed similar evolutions throughout the study for all groups with generally lower levels if older or ovariectomized. BMD increased until peak level on day 14 (day 9 for OVX), then decreased to baseline level. BMD of OVX dropped below baseline on day 28. Once normalized, BMD was similar for all groups but BMD which was significantly lower from day 14 onwards ($p<0.01$). On the contrary, Tissue Mineral Density (TMD) increased with age and ovariectomy. In all groups, TMD decreased slightly post operatively until day 6 and then plateaued. Once normalized, TMD loss post op was the same in all groups, TMD of OVX showed larger variations, most likely because there is not enough bone in these animals to get relevant data (Figure 8).

3.5 Discussion

Although age is a known confounding factor in most preclinical models and in particular in implant integration models but is often overlooked in study designs, the aim of the present study was to characterize the effect of age on implant fixation dynamics using a rat model. Relative importance of this effect can play a role when defining acceptable age ranges in a model to reach the expected statistical power. Here, the effect of age on implant integration kinetics was investigated using a longitudinal *in vivo* model with a unilateral contrast-enhanced PEEK screw in medial tibia metaphysis of rats. The rats were operated at different ages (24 weeks, 40±6 weeks, 60±5 weeks) and bone changes in the postoperative phase were closely monitored. A 33 weeks old OVX group was also used to put the age effect into perspective of the surgically induced osteoporosis.

Two distinct phases could be distinguished in the data, the early (or *post-operative*) phase (up to day 9-14 approximately) and a *maintenance* (or remodeling) phase (after 14 days) in line with the theory of osseointegration[35,36]. The static outcome parameters (BIC, BV/TV, Stiffness) mostly followed similar patterns: baseline levels were highest in young animals, decreased with age and were lowest in OVX. The downward slopes observed at day 28 suggest that a steady state was not yet reached.

Interestingly, once normalized to baseline levels, there was no difference between groups in the early phase. In the maintenance phase, the order was reverse, with old animals having more bone (relative to baseline) than young animals. OVX had maximum relative values similar to old animals but then reached the lowest levels of all groups towards the end of the study. Maximum absolute bone formation and resorption decreased with age.

In other words, younger animals have more pre-existing bone than older animals at baseline, resulting in better implant fixation, but older animals form proportionally more bone than young animals. From a biomechanical perspective, this could be interpreted as follow: the implant is

subject to some load since the screw head protrude outside the medial tibia, therefore the organism tries to fix it mechanically in bone. The absolute bone mass needed for mechanical fixation is the same in all rats, since dimensionally there are no differences, therefore rats with less bone compensate by producing more bone.

In this study bone mass and formation clearly decreased and was delayed in 40 and 60 w compared to 24 w old rats. But once normalized, we saw no difference between groups in the early phase, and higher relative values in older animals. This suggests that for non-OVX animals, baseline level (ie. pre-existing bone mass) is the main factor influencing implant integration. Once corrected for this factor, age only affects the remodeling phase of implant integration. So, this correction might allow other researchers to compare postoperative phase of study groups which do not have the same age.

Duarte *et al.* have used a similar model with titanium screws in rat tibias to study the effect of age and ovariectomy on bone density and BIC[5]. They report 51, 43 and 37% BIC in 12w, 88w and OVX rats respectively at 60 days post-op, which is in proportional agreement with our 80% 60% 40% at 28 days for 24w, 60w and OVX rats. They concluded that age affects baseline bone mass while ovariectomy affects both baseline bone mass and bone formation, which is in exact agreement with our findings studying 28 days post op only. In bone healing models, the effect of age was reported to be a delayed healing but equivalent gene expression [37,38], delayed callus maturation likely due to remodeling imbalance, but no influence on callus volume[39]. Again, in the context of healing around an implant, our observations of delayed absolute bone formation but equivalent normalized values are in total agreement with these findings.

There are limitations to our model and findings. Since implant integration is a transient process, single endpoint studies can potentially miss differences between groups. The model used here enables longitudinal, time-dependent measurements of bone distribution, changes and of the resulting implant fixation. Since this approach was first reported [25] it was successfully used to

describe bone dynamics around bisphosphonate-releasing implants [40], around infected implants [32] and in osteoporotic animals [41]. This models uses $BaSO_4$ loaded PEEK screws to prevent metallic artifacts that stainless steel or titanium would otherwise generate in tomographic reconstructions [42]. Although not as commonly used as steel and titanium, PEEK is used successfully for reconstructive and spine surgery [43,44] , therefore its use here is clinically relevant. Our normalized data qualitatively fits the data obtained with titanium screws [25] but the absolute values are specific to this material and more work is needed to extrapolate to metal implants[45].

Longitudinal *in vivo* microCT studies involve repeated x-ray exposition which may affect local metabolism, this question is still debated: the first reports showed no effect of repeated scans on bone microarchitecture [27,46], other authors recently reported an effect of repeated low dose exposure on bone[47] and on adjacent skeletal muscles which may indirectly impact local bone. Additionally, repeated prolonged anesthesia may affect overall health of the animals, particularly through circadian rhythm and reduced appetite[48]. Here since the study was comparative, all animals experienced the same doses, but the absolute values may have been affected.

In this model, the study duration was only 28 days and all the outcome measures did not reach a steady-state phase by the end of the study. Follow-up duration should be increased in future studies to allow older rats to reach steady state.

Finally, there are intrinsic limitations to the animal model itself. The lifestyle associated with standard animal housing is sedentary: it was shown that old mice and rats with access to voluntary wheel running will use the option and show no age-associated loss in osteocytes and overall better bones[49]. In this regard, our findings are for sedentary elderly and may not be valid in a more physically active model.

In conclusion, age did influence bone parameters such as BIC, BV/TV and formation/resorption around the implanted screws in absolute terms. In contrary, age did not have an effect in relative terms, suggesting that baseline bone mass is the main influencing factor. But since the two factors are covariant, when aiming to detect small treatment effects in a similar model, rats of the same age should be used. In studies where groups are made of rats with different ages, data normalization relative to baseline bone mass is necessary for meaningful interpretations.

3.6 Conflicts of interest

The authors are not compensated and there are no other institutional subsidies, corporate affiliations or funding sources supporting this work unless clearly documented and disclosed. No external funds were received in support of this work.

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3.8 References

1. Wancket LM. 2015. Animal Models for Evaluation of Bone Implants and Devices. Vet. Pathol. 52(5):842–850 [cited 2018 Jul 13] Available from: http://journals.sagepub.com/doi/pdf/10.1177/0300985815593124.

2. Schindeler A, Mills RJ, Bobyn JD, Little DG. 2018. Preclinical models for orthopedic research and bone tissue engineering. J. Orthop. Res. 36(3):832–840 [cited 2018 Jul 13] Available from: http://doi.wiley.com/10.1002/jor.23824.

3. Freedman LP, Cockburn IM, Simcoe TS. 2015. The economics of reproducibility in preclinical research. PLoS Biol. 13(6):1–9 [cited 2018 Jun 27] Available from: http://journals.plos.org/plosbiology/article/file?id=10.1371/journal.pbio.1002165&type=printable.

4. Jackson SJ, Andrews N, Ball D, et al. 2017. Does age matter? The impact of rodent age on study outcomes. Lab. Anim. 51(2):160–169 [cited 2018 Jun 27] Available from: http://www.ncbi.nlm.nih.gov/pubmed/27307423.

5. Duarte PM, Gonçalves PF, Casati MZ, et al. 2005. Age-related and surgically induced estrogen deficiencies may differently affect bone around titanium implants in rats. J. Periodontol. 76(9):1496–501 Available from: http://www.joponline.org/doi/10.1902/jop.2005.76.9.1496.

6. Ross BS, Zarb GA. 1998. Osseointegration of Oral Implants in Older and Younger Adults. Int. J. Oral Maxillofac. Implants 13(4):492--499 [cited 2017 Aug 15] Available from: http://medlib.yu.ac.kr/eur_j_oph/ijom/IJOMI/ijomi_13_492.pdf.

7. Piarulli G, Rossi A, Zatti G. 2013. Osseointegration in the elderly. In: Aging Clinical and Experimental Research. Springer International Publishing. p 59–60 [cited 2017 Aug 15] Available from: http://link.springer.com/10.1007/s40520-013-0103-0.

8. Drinias V, Granström G, Tjellström A. 2007. High Age at the Time of Implant Installation is Correlated with Increased Loss of Osseointegrated Implants in the Temporal Bone. Clin. Implant Dent. Relat. Res. 9(2):94–99 [cited 2017 Aug 15] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17535333.

9. Boskey AL, Coleman R. 2010. Aging and Bone. J. Dent. Res. 89(12):1333–1348 [cited 2018 Jun 24] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20924069.

10. Halvorson TL, Kelley LA, Thomas KA, et al. 1994. Effects of bone mineral density on pedicle screw fixation. Spine (Phila Pa 1976) 19(21):2415–2420 Available from: http://www.ncbi.nlm.nih.gov/pubmed/7846594.

11. Gabet Y, Kohavi D, Voide R, et al. 2010. Endosseous implant anchorage is critically dependent on mechanostructural determinants of peri-implant bone trabeculae. J. Bone Miner. Res. 25(3):575–583 [cited 2017 Aug 10] Available from: http://doi.wiley.com/10.1359/jbmr.090808.

12. Nilsson BE, Edwards P. 1969. Age and fracture healing: a statistical analysis of 418 cases of tibial shaft fractures. Geriatrics 24(2):112–117 [cited 2018 Jun 28] Available from: http://www.ncbi.nlm.nih.gov/pubmed/5762691.

13. Meyer MH, Meyer RA. 2007. Genes with Greater up-regulation in the fracture callus of older rats with delayed healing. J. Orthop. Res. 25(4):488–494.

14. Meakin LB, Udeh C, Galea GL, et al. 2015. Exercise does not enhance aged bone's impaired response to artificial loading in C57Bl/6 mice. Bone 81(July):47–52 Available from: http://dx.doi.org/10.1016/j.bone.2015.06.026.

15. Baht GS, Silkstone D, Vi L, et al. 2015. Exposure to a youthful circulation rejuvenates bone repair through modulation of β-catenin. Nat. Commun. 6(1):7131 [cited 2018 Jun

28] Available from: http://www.ncbi.nlm.nih.gov/pubmed/25988592.

16. Dutta S, Sengupta P. 2016. Men and mice: Relating their ages. Life Sci. 152:244–248 Available from: http://dx.doi.org/10.1016/j.lfs.2015.10.025.

17. Sengupta P. 2013. The Laboratory Rat: Relating Its Age With Human's. Int. J. Prev. Med. 4(6):624–30 [cited 2018 Jul 31] Available from: http://www.ncbi.nlm.nih.gov/pubmed/23930179.

18. Netto CC, Vieira VCC, Marinheiro LPF, et al. 2012. Are skeletally mature female rats a suitable model to study osteoporosis? Arq. Bras. Endocrinol. Metabol. 56(4):259–264 Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0004-27302012000400007&lng=en&tlng=en.

19. Somerville JM, Aspden RM, Armour KE, et al. 2004. Growth of C57Bl/6 Mice and the Material and Mechanical Properties of Cortical Bone from the Tibia. Calcif. Tissue Int. 74(5):469–475 [cited 2018 Jun 28] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14961209.

20. Mavrogenis AF, Dimitriou R, Parvizi J, Babis GC. 2009. Biology of implant osseointegration. J. Musculoskelet. Neuronal Interact. 9(2):61–71 Available from: http://www.ismni.org/jmni/pdf/36/01MAVROGENIS.pdf.

21. Dhert WJA, Thomsen P, Blomgren AK, et al. 1998. Integration of press-fit implants in cortical bone: A study on interface kinetics. J. Biomed. Mater. Res. 41(4):574–583.

22. Åstrand J, Aspenberg P. 2004. Topical, single dose bisphosphonate treatment reduced bone resorption in a rat model for prosthetic loosening. J. Orthop. Res. 22(2):244–249 [cited 2017 Aug 10] Available from: http://www3.interscience.wiley.com/journal/112218239/abstract?CRETRY=1&SRETR

Y=0.

23. Mair B, Tangl S, Feierfeil J, et al. 2009. Age-related efficacy of parathyroid hormone on osseointegration in the rat. Clin. Oral Implants Res. 20(4):400–405 [cited 2017 Aug 15] Available from: http://doi.wiley.com/10.1111/j.1600-0501.2008.01658.x.

24. Back DA, Pauly S, Rommel L, et al. 2012. Effect of local zoledronate on implant osseointegration in a rat model. BMC Musculoskelet. Disord. 13:42 [cited 2017 Aug 14] Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3323428/pdf/1471-2474-13-42.pdf.

25. Stadelmann VA, Conway CM, Boyd SK. 2013. In vivo monitoring of bone–implant bond strength by microCT and finite element modelling. Comput. Methods Biomech. Biomed. Engin. 16(9):993–1001 Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22289208&retmode=ref&cmd=prlinks.

26. Jee WS, Yao W. 2001. Overview: animal models of osteopenia and osteoporosis. J. Musculoskelet. Neuronal Interact. 1(3):193–207 [cited 2018 Jun 28] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15758493.

27. Klinck RJ, Campbell GM, Boyd SK. 2008. Radiation effects on bone architecture in mice and rats resulting from in vivo micro-computed tomography scanning. Med. Eng. Phys. 30(7):888–895 [cited 2017 Oct 17] Available from: http://linkinghub.elsevier.com/retrieve/pii/S1350453307001889.

28. Bouxsein ML, Boyd SK, Christiansen BA, et al. 2010. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J. Bone Miner. Res. 25(7):1468–1486.

29. Schulte FA, Lambers FM, Kuhn G, Müller R. 2011. In vivo micro-computed tomography allows direct three-dimensional quantification of both bone formation and bone resorption parameters using time-lapsed imaging. Bone 48(3):433–442 [cited 2011 Oct 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20950723.

30. Waarsing JH, Day JS, Van Der Linden JC, et al. 2004. Detecting and tracking local changes in the tibiae of individual rats: A novel method to analyse longitudinal in vivo micro-CT data. Bone 34(1):163–169 [cited 2018 Jul 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14751574.

31. Engelke K, van Rietbergen B, Zysset P. 2016. FEA to Measure Bone Strength: A Review. Clin. Rev. Bone Miner. Metab. 14(1).

32. Stadelmann VA, Potapova I, Camenisch K, et al. 2015. In Vivo MicroCT Monitoring of Osteomyelitis in a Rat Model. Biomed Res. Int. 2015:587857 Available from: http://www.hindawi.com/journals/bmri/2015/587857/.

33. Pistoia W, van Rietbergen B, Lochmüller E-M, et al. 2002. Estimation of distal radius failure load with micro-finite element analysis models based on three-dimensional peripheral quantitative computed tomography images. Bone 30(6):842–8 Available from: http://www.ncbi.nlm.nih.gov/pubmed/12052451.

34. R Core team. 2015. R Core Team. R A Lang. Environ. Stat. Comput. R Found. Stat. Comput. , Vienna, Austria. ISBN 3-900051-07-0, URL http//www.R-project.org/. 55:275–286 Available from: http://www.mendeley.com/research/r-language-environment-statistical-computing-96/%5Cnpapers2://publication/uuid/A1207DAB-22D3-4A04-82FB-D4DD5AD57C28.

35. Raghavendra S, Wood MC, Taylor TD. [date unknown]. Early wound healing around endosseous implants: a review of the literature. Int. J. Oral Maxillofac. Implants

20(3):425–31 [cited 2018 Jun 28] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15973954.

36. Puleo D., Nanci A. 1999. Understanding and controlling the bone–implant interface. Biomaterials 20(23–24):2311–2321 [cited 2018 Jun 28] Available from: https://www.sciencedirect.com/science/article/pii/S014296129900160X.

37. Meyer RA, Desai BR, Heiner DE, et al. 2006. Young, adult, and old rats have similar changes in mRNA expression of many skeletal genes after fracture despite delayed healing with age. J. Orthop. Res. 24(10):1933–1944 [cited 2018 Apr 23] Available from: http://doi.wiley.com/10.1002/jor.20124.

38. Hebb JH, Ashley JW, McDaniel L, et al. 2018. Bone healing in an aged murine fracture model is characterized by sustained callus inflammation and decreased cell proliferation. J. Orthop. Res. 36(1):149–158 [cited 2018 Apr 23] Available from: http://doi.wiley.com/10.1002/jor.23652.

39. Mehta M, Strube P, Peters A, et al. 2010. Influences of age and mechanical stability on volume, microstructure, and mineralization of the fracture callus during bone healing: Is osteoclast activity the key to age-related impaired healing? Bone 47(2):219–228 Available from: http://linkinghub.elsevier.com/retrieve/pii/S8756328210012809.

40. Kettenberger U, Latypova A, Terrier A, Pioletti DP. 2015. Time course of bone screw fixation following a local delivery of Zoledronate in a rat femoral model - A micro-finite element analysis. J. Mech. Behav. Biomed. Mater. 45(2):22–31 [cited 2018 Mar 31] Available from: www.elsevier.com/locate/jmbbm.

41. Li Z, Kuhn G, Schirmer M, et al. 2017. Impaired bone formation in ovariectomized mice reduces implant integration as indicated by longitudinal in vivo micro-computed tomography. PLoS One 12(9):e0184835 Available from:

http://dx.plos.org/10.1371/journal.pone.0184835.

42. Boas FE, Fleischmann D. 2012. CT artifacts: causes and reduction techniques. Imaging Med. 4(2):229–240 [cited 2018 Jul 13] Available from: http://www.edboas.com/science/CT/0012.pdf.

43. Schwitalla A, Müller W-D. 2013. PEEK Dental Implants: A Review of the Literature. J. Oral Implantol. 39(6):743–749 [cited 2018 Jun 29] Available from: http://www.joionline.org/doi/abs/10.1563/AAID-JOI-D-11-00002.

44. Schimmel JJP, Poeschmann MS, Horsting PP, et al. 2016. PEEK Cages in Lumbar Fusion Mid-term Clinical Outcome and Radiologic Fusion. Clin. Spine Surg. 29(5):E252–E258 [cited 2018 Jun 29] Available from: http://www.ncbi.nlm.nih.gov/pubmed/27196005.

45. Shimizu T, Fujibayashi S, Yamaguchi S, et al. 2017. In vivo experimental study of anterior cervical fusion using bioactive polyetheretherketone in a canine model. PLoS One 12(9):e0184495 [cited 2018 Jul 13] Available from: http://dx.plos.org/10.1371/journal.pone.0184495.

46. Beck A, Woods S, Lansdowne JL, Arens D. 2013. The effects of multiple high-resolution peripheral quantitative computed tomography scans on bone healing in a rabbit radial bone defect model. Bone 56(2):312–319 [cited 2018 Jul 13] Available from: http://www.ncbi.nlm.nih.gov/pubmed/23827347.

47. Lima F, Swift JM, Greene ES, et al. 2017. Exposure to Low-Dose X-Ray Radiation Alters Bone Progenitor Cells and Bone Microarchitecture. Radiat. Res. (August):RR14414.1 Available from: http://www.ncbi.nlm.nih.gov/pubmed/28771086%0Ahttp://www.rrjournal.org/doi/10.1667/RR14414.1.

48. Kärkela J, Vakkuri O, Kaukinen S, Huang WQ. 2002. The influence of anaesthesia and surgery on the circadian rhythm of melatonin. Acta Anaesthesiol. Scand. 46(1):30–36 [cited 2018 Jul 13] Available from: http://doi.wiley.com/10.1034/j.1399-6576.2002.460106.x.

49. Tiede-Lewis LAM, Xie Y, Hulbert MA, et al. 2017. Degeneration of the osteocyte network in the C57BL/6 mouse model of aging. Aging (Albany. NY). 9(10):2187–2205 [cited 2018 Jun 29] Available from: http://www.ncbi.nlm.nih.gov/pubmed/29074822.

3.9 Tables

Group n°	Group name	N	Age at screw implantation (weeks)	Ovariectomy
1	**24w**	**6**	**24±1**	**No**
2	**40w**	**11**	**40±6**	**No**
3	**60w**	**8**	**60±5**	**No**
4	**OVX**	**5**	**33±1**	**Yes**

Table 1 - group distribution

3.10 Figures

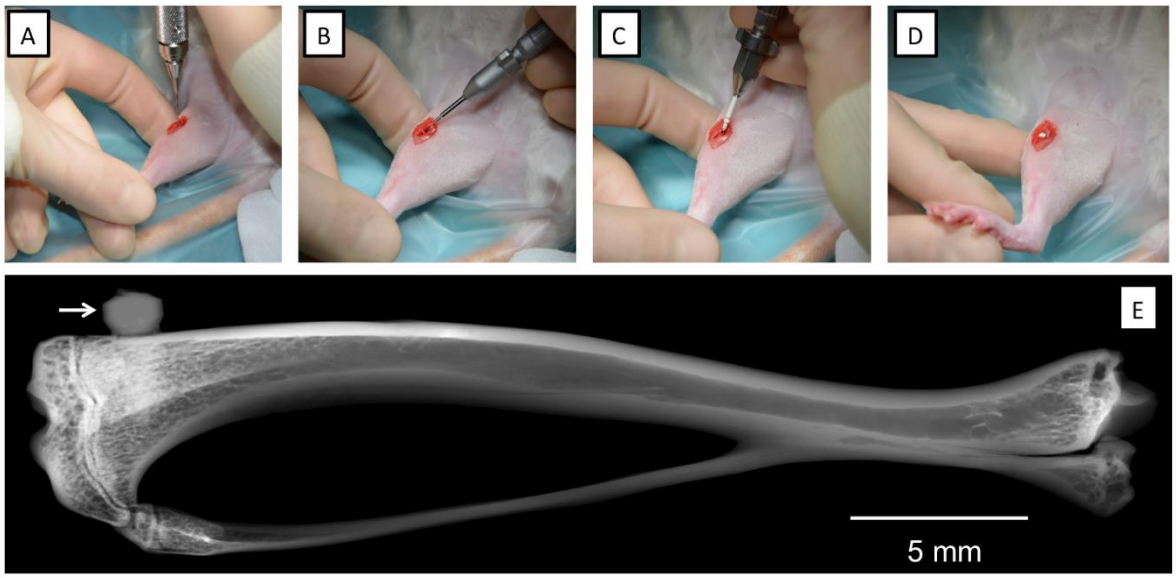

Figure 1 - Implantation of the screw in the medio-proximal tibia. (A) Drilling of the hole (B) Tapping the thread (C) Screw insertion, screw extension still attached (D) Inserted screw, extension is cut off. (E) Radiography of an explanted operated tibia showing the inserted screw in the proximal bone (arrow).

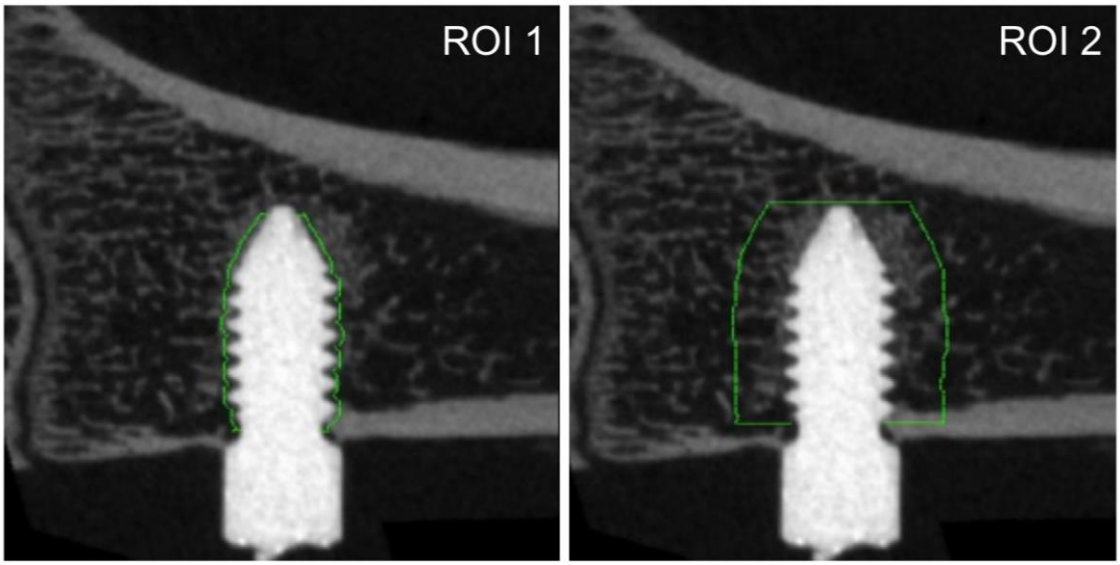

Figure 2 – Two Regions of interest are automatically generated in the trabecular bone. ROI 1 at screw surface for bone-implant contact (BIC) and ROI 2 within peri-implant trabecular bone for peri-implant bone microstructure and remodeling.

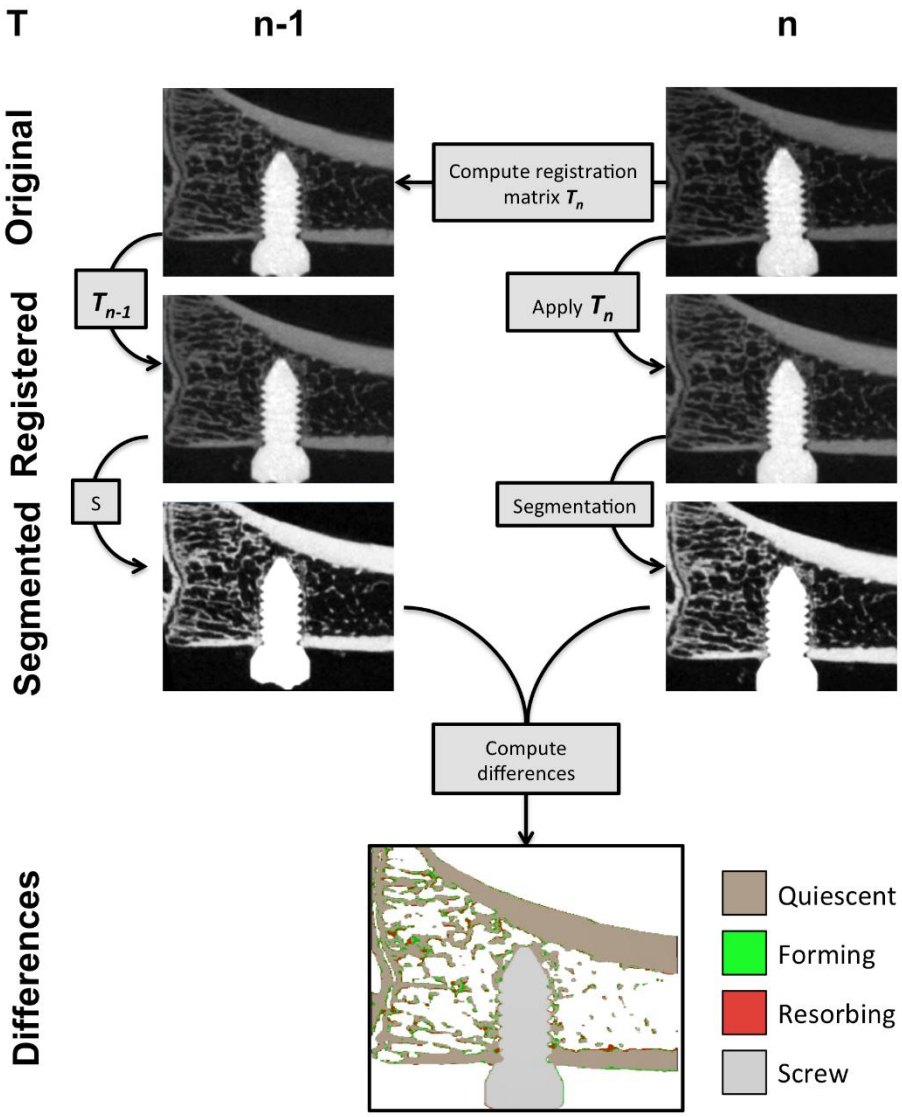

Figure 3 - Scheme of the method to compute bone formation and bone resorption from two consecutive scans. A scan a timepoint t_n is aligned to the previous scan at t_{n-1} using rigid registration. The t_{n-1} and t_n scans are segmented and overlapped to generate a difference map. Bone formation, resorption and quiescence are labeled from this map.

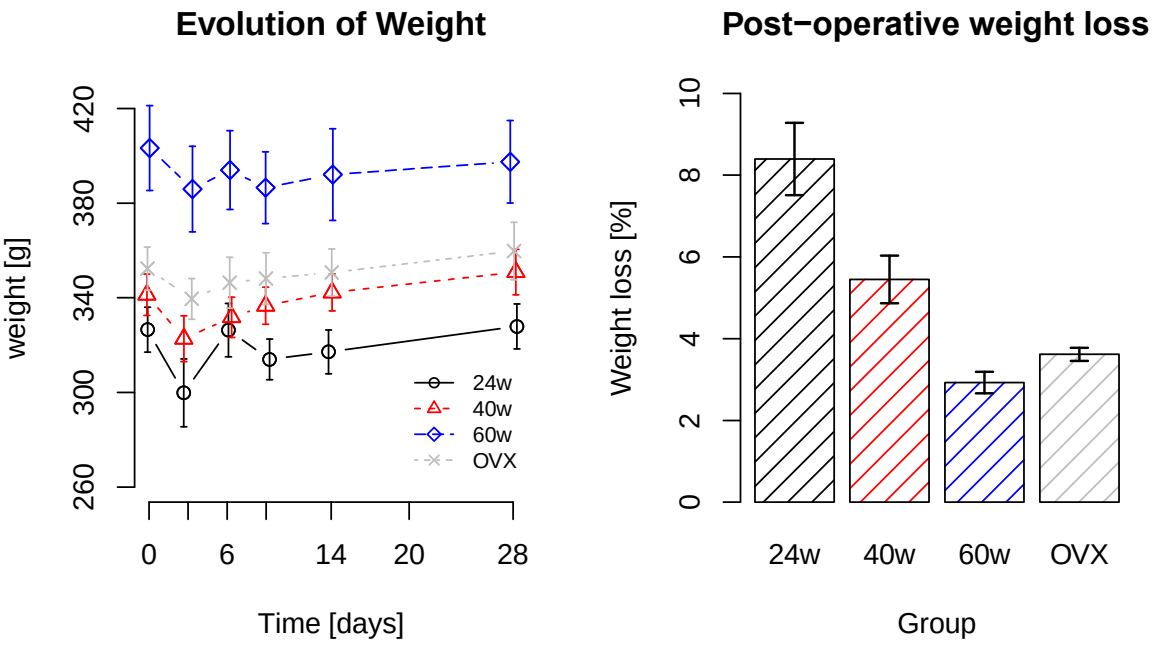

Figure 4 – (Left) Evolution of animal weights during the study and (right) post-operative weight loss in the first 3 days following screw implantation.

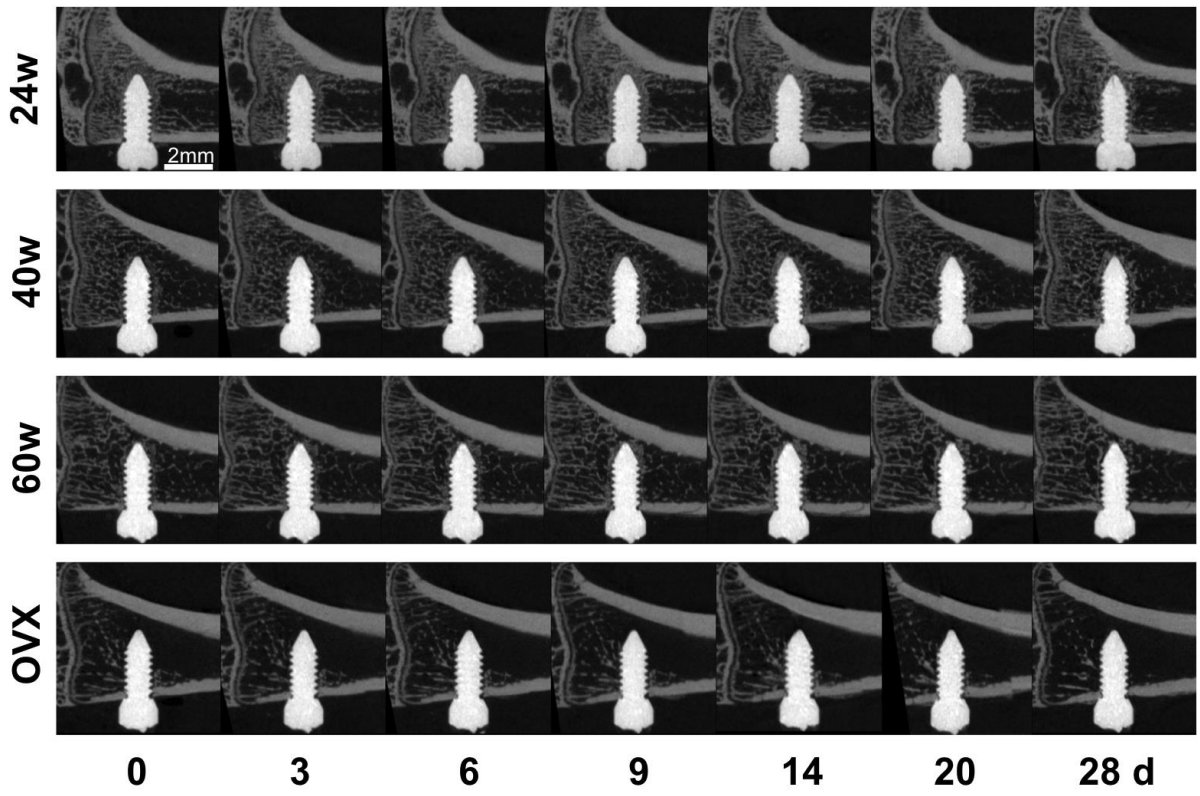

Figure 5 - Longitudinal time series showing the center microCT slice at every timepoint, for the one animal per group with BV/TV closest to the group's median value

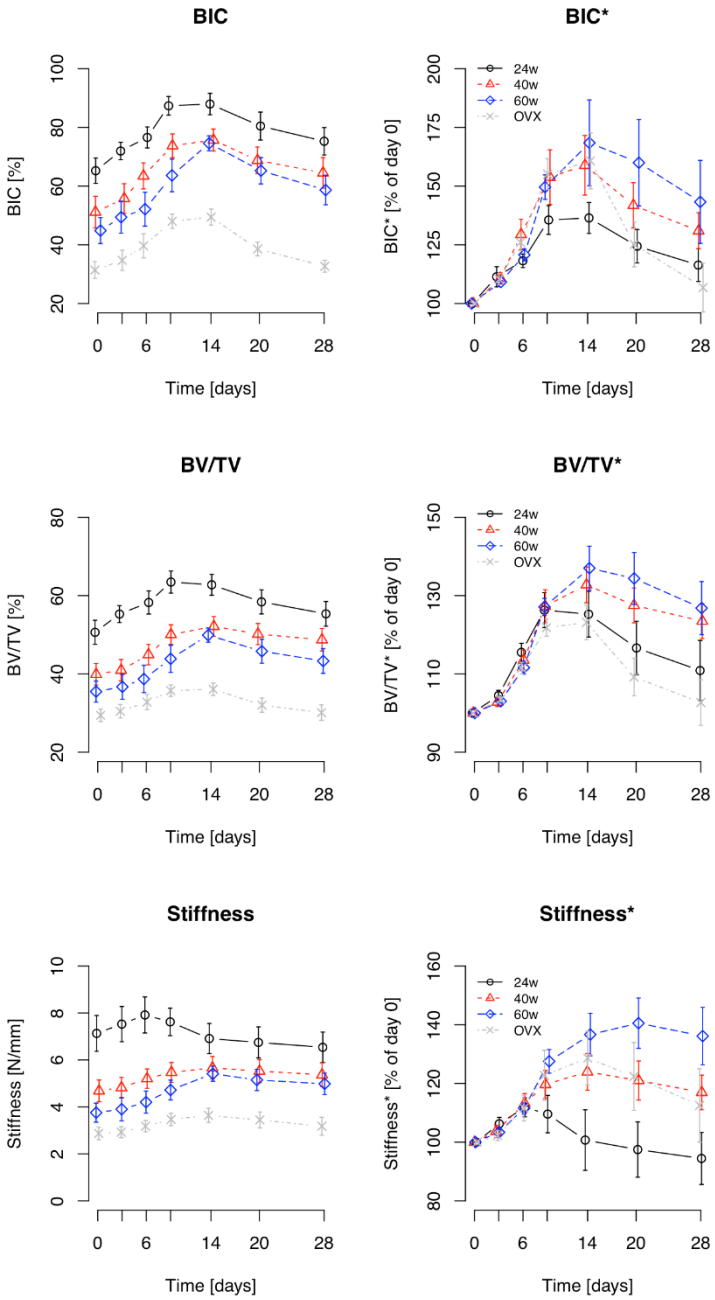

Figure 6 - Evolutions of bone-implant contact (BIC), bone fraction (BV/TV) and FEA stiffness over time. Left column shows absolute values and right column shows relative data normalized to d0.

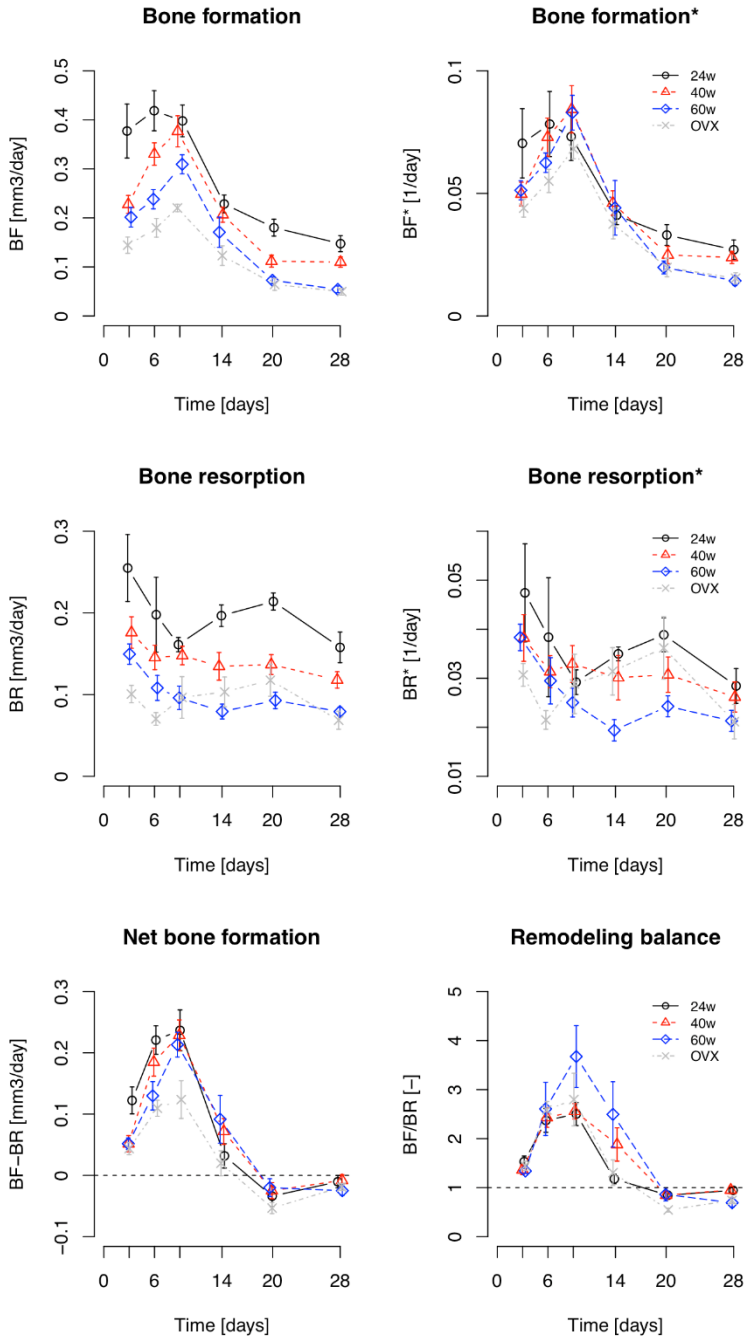

Figure 7– Evolutions of Bone Formation and Bone Resorption, absolute (left) and normalized (to d0) data (right), and evolutions of Net Bone Formation (BF-BR) and Remodeling Balance (BF/BR)

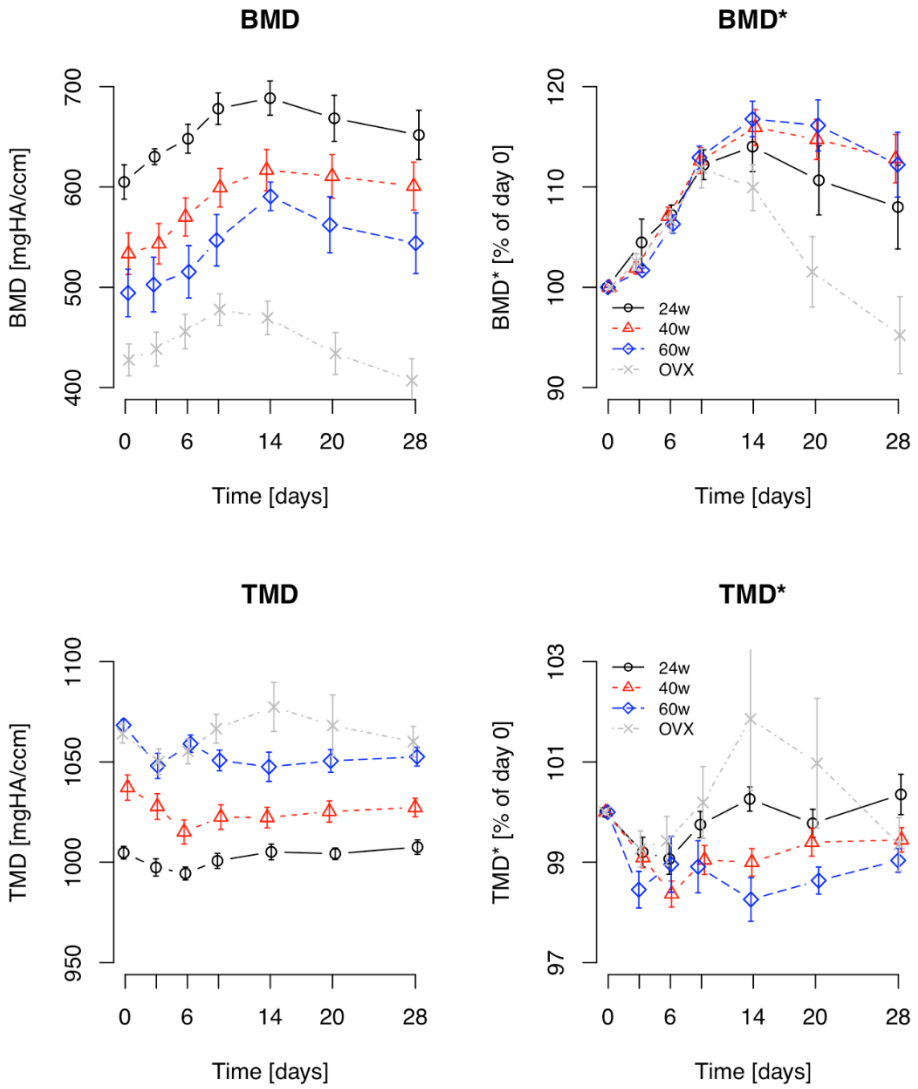

Figure 8 Evolutions of Bone Mineral Density (BMD) and Tissue Mineral Density (TMD) in ROI2. Absolute data on the left, normalized data (to d0) on the right.

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